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Analysis of a strain of green algae resistant to cadmium

Morphological and Physiological Changes exhibited by a Cd-resistant *Dictyosphaerium chlorelloides* Strain and its Cadmium Removal Capacity

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Wild-type Dc1M previoexposed to Cd causes the appearance of resistant cells, Dc^{CdR100} cells exhibit morphological and photosynthetic changes, Dc^{CdR100} cells were found to have good accumulation properties for cadmium, This fact enables the use of these Dc^{CdR100} cells in bioremediation processes

ABSTRACT

Changes induced on freshwater microalga *Dictyosphaerium chlorelloides* (Dc^{wt}), acclimated in the laboratory until their survival in culture media enriched with cadmium 100 µM, have been

studied. Cadmium removal by living cells of this Cd-resistant (Dc^{CdR100}) strain was tested in cultures exposed to Cd 100 µM during 30 days. Cell dimensions were measured under light microscopy, and cell growth was studied. Photosynthetic yield (Φ_{PSII}) was analyzed and the photosynthetic oxygen development and respiration response was obtained. Results show that Dc^{CdR100} strain exhibited significant cell morphology changes in comparison to Dc^{wt} cells, which affected both surface and cell biovolume. Malthusian fitness analysis showed that Dc^{CdR100} strain living in Cd-enriched culture had developed a lower capacity of nearly 50% growth, and its photosynthetic oxygen development and respiration response was significantly reduced in both light and dark photosynthetic phases. Dc^{CdR100} strain showed a very high capacity to remove cadmium from the aquatic environment (over 90%), although the most of the heavy metal removed (≈70%) is adhered to the cell wall. These specific characteristics of Dc^{CdR100} cells suggest the possibility of using this strain in conjunction with Dc^{wt} strain as bioelements into a dual-head biosensor, and in bioremediation processes on freshwater polluted with Cd.

Key Words

Dictyosphaerium chlorelloides; Cd-resistant microalgae; morphological changes; photosynthesis activity; cadmium removal.

Introduction

The right balance of aquatic ecosystems is a key element in environmental studies. As water is an essential resource, its quality needs early warning systems for online and on-site monitoring contamination. Therefore, cell-based sensors as algal biosensors are increasingly replacing to chemical methods (Naessens et al., 2000) due to its usability, low cost and immediacy, among other advantages (Naessens et al., 2000; Sanders et al., 2001; Védrine et al., 2003).

Microalgae are used as bioindicators in the field of ecotoxicological studies. Because of its ubiquity, short life cycles, high sensitivity and ease of growth, they are used regularly in ecotoxicological screening of contaminated freshwater (Lewis, 1995). As primary producers, microalgae have an important role in nutrient cycling and balance of aquatic ecosystems (Raja et al., 2008).

The development of algae-based biosensors required to achieve high sensitivity, improved signal reception and optimization of cell immobilization methods. However, although these sensors have sufficient sensitivity, most of them exhibit low specificity and limited over a range of pollutants (D'Souza, 2001). Therefore, relatively few laboratory biosensors have become commercially viable methods (Bengtson Nash et al., 2005). Furthermore, use of highly selective biosensors can be complicated given the large number of potentially environmentally hazardous substances (Podola et al., 2004). Therefore, a combination of non-selective biosensor capable of detecting a wide range of contaminants, together with a selective biosensor capable of detecting a specific pollutant would be the jump for the gap that separates academic research from field applications.

Present alternatives to increase specificity in biosensors include expensive genetic modifications of these organisms, such as microalgae (Costas et al., 2001; López-Rodas et al., 2001; García-Villada et al., 2002). The most preferred biological process to assess the effects of contaminants on microalgae is the photosynthetic activity, estimated by chlorophyll a fluorescence of photosystem II (PSII), or oxygen production (Altamirano et al., 2004).

Large number of papers have reported high sensitivity of microalgae to heavy metals (Rai et al 1981; García-Villada et al 2004) and however some microalgal strains tolerate high concentrations of these contaminants through physiological and genetic mechanisms. This adaptive capacity displayed by some microalgae may have originated in previous exhibitions (Corradi et al 1995; Corradi et al., 1998) and could play an important role on the biological effects of heavy metal pollution.

Cadmium is a nonessential element that can be toxic and carcinogenic. On a global scale, the ratio anthropogenic to natural emissions of cadmium is approximately 7:1. Much of the cadmium added to aquatic systems accumulates in sediments where it presents a risk to benthic biota and under certain conditions may reenter the water column (Wright and Welbourn, 1994).

Previous studies demonstrated that wild-type *Dictyosphaerium chlorelloides* was very sensitive to the effect of heavy metals, but it was verified that after destruction of most algae following to these chromium exposures, some rare, resistant algal variants are able to grow in an environment with high levels of chromium (Sánchez-Fortún et al., 2009; D'ors et al., 2010).

For this reason, the aims of this work were to estimate the toxic effect of cadmium on Cd-sensitive (Dc^{wt}) and Cd-resistant (Dc^{CdR100}) strains of the green algae *D. chlorelloides*, and to study the specific tolerant level obtained by the Cd-resistant clone respect to the wild type strain.

With these data it will be possible to evaluate the potential Dc^{CdR100} strain to remove cadmium from aquatic ecosystems.

Material and Methods

Microalgae culture conditions

Cadmium sensitive (Dc^{wt}) and resistant (Dc^{CdR100}) strains of *Dyctiosphaerium chlorelloides*, from the algal culture collection of the Environmental Toxicology Laboratory (Toxicology and Pharmacology Department, UCM, Madrid, Spain), were used in these assays. Dc^{wt} strain (wild type Dc1M) axenically grew in culture flasks (Greiner 25 cm^2 50 ml, Bio-One, Longwood, NJ, USA) with 20 ml of BG-11 medium (pH 7.1; Sigma, Aldrich Chemie, Taufkirchen, Germany), whereas Dc^{CdR100} strain, previously obtained from the wild type Dc1M strain by acclimation under laboratory conditions in cadmium-enriched medium, was cultured in BG-11 medium supplied with 29 mg L^{-1} cadmium chloride ($CdCl_2$; Sigma, Aldrich Chemie, Taufkirchen, Germany) which is equivalent to Cd 100 µM concentration. Both strains were maintained at 21 ºC under continuous light of 60 µmol m^{-2} s^{-1} over the 400 to 700 nm waveband. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculums to fresh medium once a fortnight. Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures. In order to unify experimentation criteria, all tests were performed while both microalgal strains were in exponential growth phase.

Biovolume Measurement

Cell samples from Dc^{wt} and Dc^{CdR100} strains were selected and analyzed using light microscopy. Samples were observed with an Olympus CK-2 microscope at x400 magnification in a Neubauer counting chamber.

Linear dimensions were measured according to Sun and Liu (2003). Biovolume (V) was obtained applying the following formula:

$V = a^3 * \pi/6$

where a is the cell diameter. Fifty individual cells of each strain were measured, and the information were then input into a Microsoft Excel worksheet which V±sd were calculated.

Malthusian Fitness Assay

Malthusian fitness (m) was calculated in accordance with Crow and Kimura (1970) in eight replicates of each *D. chlorelloides* strain as well as in eight controls, using the following equation:

$m=\log_e(N_t/N_0)/t$

where t is the number of days elapsed since the beginning of the experiment, and N_0-N_t are cell numbers at the start and the selected days of experiment, respectively.

Photosynthesis Activity

Photosynthetic yield (Φ_{PSII}) was analyzed by means of the dual-channel PAM chlorophyll fluorometer (ToxY-PAM, Heinz Walz GmbH, Germany). The ToxY-PAM dual-channel yield analyser obtains highly sensitive measurements of effective quantum yield of the algae pigment

system II centres via assessment of the chlorophyll fluorescence yield and the saturation pulse method (Schreiber et al 2002).

Effective quantum yields (Y1 and Y2) were calculated from the fluorescence parameters F1, F2, Fm1 and Fm2 according to the equation:

Y = (Fm - F) / Fm (Genty et al. 1989)

where Fm corresponding to the maximal fluorescence yield measured during a saturation pulse, and F corresponding to the fluorescence yield measured shortly before application of a saturation pulse. Values are expressed as Dc^{CdR100} Φ_{PSII} inhibition (%) respect to values obtained from Dc^{wt}.

Gross photosynthesis rate (P_g) was estimated from the formula:

$P_g = P_n + R$

where P_g corresponding to the oxygen production rate of photosystem II, R (respiration) corresponding to the process by which in the presence of light microalgal cells consume oxygen and releases carbon dioxide, and P_n (net photosynthesis rate) is defined as the difference between P_g and R. The oxygen values were obtained employing a Clark-type electrode. Dissolved O_2 was measured in a 1 ml reaction chamber from a Chlorolab 2 System (Hansatech, Norfolk, UK). Chlorolab 2 provides a system for the study of respiration and photosynthesis from liquid samples under automated illumination from red (660 nm) LED light. In the toxicity assays, measurements were taken at 21 °C and 700 μmol m^{-2} s^{-1} irradiance.

The Φ_{PSII} activity was monitored on the software package ToxyWin v1.14 (Heinz Waltz GmbH, Germany), and oxygen measurements obtained both in darkness and in light conditions, were exported to a computerized chart recorder (O_2view, Hansatech, Norfolk, UK).

Determination of cadmium removed

The Cd removed by Dc^{CdR100} strain was evaluated using a modified method described by Stauber and Florence (1985). Total Cd in cells was determined from Dc^{CdR100} cultures into 20 ml culture flasks with initial cell densities adjusted to 10^4 cells ml^{-1}. Cultures were maintained during 30 days exposed to Cd^{2+} 100 µM under axenic growth up to a final cell concentration of $20x10^6$ cells ml^{-1}. From these cultures, 4 ml aliquots of each were filtered through two superposed 1.2 µm MF-Millipore filters. Thus samples containing final concentrations of $80x10^6$ cells ml^{-1} were obtained. Filters were separately digested at room temperature for 24 h with 1 ml of 15 M HNO_3 and 0.5 ml $HClO_4$. Cadmium was measured in both filters and the lower filter was used as blank.

Intracellular cadmium was measured fromsamples obtained by centrifugation at 4000xg for 5 min. Each pellet, contained a final cell concentration of $80x10^6$ cells ml^{-1}, was resuspended for 20 min in 25 ml of a solution containing 0.02 M EDTA dissolved in deionized water. Afterwards, cells were centrifuged and washed twice with deionized water. The EDTA washing removed cadmium adsorbed onto the cell surface, thereby allowing only intracellular cadmium to be measured. The washed pellet was digested as in total Cd determination.

Digested samples were brought to final volume of 5 ml with Milli-Q water. Cadmium present in the samples was measured by inductively coupled plasma mass spectrometry (ICP-MS) System in a 4300DV Perkin-Elmer ICP-MS (Perkin-Elmer Inc., Waltham, MA, USA), according to Ward and Savage (1994). Cadmium bioadsorbed onto the cell surface was determined by subtracting the intracellular cadmium concentration from the total cadmium removed (bioadsorbed Cd = Total Cd - Intracellular Cd). The Cd removal percentage (%) was calculated on the basis of cadmium added to cultures (100 µM).

Data Analysis

Eight culture replicates were used for each experiment (n=8). Statistical analysis was performed using the computer software package GraphPad Prism v5.0 (Graph-Pad Software Inc., USA). A t-test analysis was used to compare the average between two groups ($p<0.05$).

Results

The results show that cells from Dc^{CdR100}, obtained from Dc^{wt} populations growing under laboratory conditions in BG-11 medium supplemented with Cd 100µM, exhibit significant changes in their cell morphology in comparison to Dc^{wt} cells. Figure 1 shows a significant reduction of cell size in Dc^{CdR100} strain compared to Dc^{wt}. These changes affect both surface and cell biovolume.

Measurements made in a relevant number of Dc^{wt} and Dc^{CdR100} cells (n=50) displayed a significant reduction of cell surface exposed to the external environment, obtaining values of 1.82±0.59 μm^2 and 0.71±0.22 μm^2 to Dc^{wt} and Dc^{CdR100} cells, respectively. Similar results have been obtained to determine cell biovolume in both *D. chlorelloides* strains, obtaining values of 0.24±0.11 μm^3 and 0.06±0.03 μm^3 to Dc^{wt} and Dc^{CdR100} cells, respectively. In both cases, there are statistically significant differences ($p<0.0001$) between the two *D. chlorelloides* strains (Figure 2).

Malthusian fitness analysis shows that Dc^{wt} and Dc^{CdR100} cells show m values of 2.53±0.55 and 1.65±0.33 doublings d^{-1} respectively. This reduced cell growth ability represents statistically significant differences ($p<0.001$) between the two strains (Figure 3).

Chlorophyll fluorescence measurements, obtained by mean the dual-channel PAM fluorometer (ToxY-PAM) are summarized in Table 1. When Dc^{wt} strain was confronted with itself, Y1 and Y2 values were similar. However, when Dc^{wt} (channel 2, reference) and Dc^{CdR100} (channel 1, sample) were confronted, the effective quantum yield in channel 1 (Y1=0.15±0.01) was significantly lower than channel 2 (Y2=0.44±0.01), obtaining a Φ_{PSII} inhibition percentage of 64.87±0.91 % in Dc^{CdR100} samples compared to Dc^{wt} samples. These results demonstrates that the changes in Dc^{CdR100} strain directly or indirectly affect the complex process of photosynthesis and thus lower the quantum yield of energy conversion at the photosynthetic reaction centers.

Analysis of production and consumption oxygen levels exhibited by both *D. chlorelloides* strains is summarized in Table 2. Oxygen balance displayed on gross photosynthesis activity of Dc^{CdR100} strain is 25.95 ± 3.91% lower than that exhibited by the wild type Dc^{wt}. When the results are broken down into those obtained during light (Pn) and dark (R) phases, can be seen that Dc^{CdR100} strain shows a higher deficit in O_2 consumption (55.81±7.52 %) than O_2 production (25.64±3.59 %), respect to values exhibited by Dc^{wt}

Our results show that Dc^{CdR100} strain cells are able to bioaccumulate and bioabsorb cadmium, allowing it to be removed from the medium. This microalgal strain showed a high ability for cadmium removal. Table 3 shows the cadmium removed from Dc^{CdR100} cultures after 30 days. Part of the total cadmium removed by cells was accumulated intracellularly and another part was adsorbed onto the cell surface. The amount of intracellular Cd^{2+} was 29.18 µM, while bioadsorbed Cd^{+2} was 62.45 µM. Thus, in cultures exposed to Cd 100µM the uptake of cadmium reached a value of $1.29x10^{-4}$ µg $cell^{-1}$ in 30 days. These data show that Dc^{CdR100} strain has a very

high capacity to remove cadmium from the aquatic environment (over 90%), although the most of the heavy metal removed (≈70%) is adhered to the cell wall.

Discussion

The aim of our study was to analyze the characteristics and degree of specificity exhibited by a Cd-resistant *D. chlorelloides* strain, obtained from the Cd-senstive wild type strain long time grown in BG-11 culture medium with cadmium-enriched progressively until achieving a permanent population living in an environment containing Cd 100 µM, to be used in bioremediation processes and as bioelement in a whole-cell biosensor system. Cadmium is one of the most toxic metals with no described biological function. This heavy metal can produce serious hazards to aquatic organisms, including microalgae.

The significant effect of Cd 100 µM on Malthusian fitness of Dc^{CdR100}, respect to those obtained in Dc^{wt} populations living in growing in BG-11 without cadmium, suggests that the survival of this Cd-resistant strain could only be achieved by some kind of adaptation to these adverse conditions. Thus, after further incubation for several months, some cultures showed growth of cells that were resistant to the effect of Cd. The key to understanding adaptation of the wild type *D. chlorelloides* to these extremely adverse conditions is to analyze these rare algal variants that occur after the massive destruction of the sensitive cells by Cd.

Our results demonstrate that acclimatization of the wild type *D. chlorelloides* strain to live in a culture medium highly contaminated with Cd (Dc^{CdR100}) induced morphological changes mainly related to cell size. This fact are in agreement with other authors which have suggested that cell morphology of microalgal populations exposed to highly contaminated environments

rapidly evolving by successive beneficial mutations spreading rapidly through natural selection (Lenski and Travisano, 1994; Elena et al., 1996). This specific lower cell size exhibited by Dc^{CdR100} is according with the results obtained in *D. chlorelloides* strains resistant to TNT (López-Rodas et al. 2006), Cr^{+6} (D'ors et al. 2010) or Cr^{+3} (Pereira et al., 2013). However, no cell modifications were observed in *Scenedesmus intermedius* (Baos et al. 2002) or *Microcystis aeruginosa* (García-Villada et al. 2004) strains which survived in highly polluted aquatic environments. These differences could be due to the existence of different strategies exhibited by these organisms to survive in extreme environments.

Additionally, we propose the hypothesis based on the concept already tested by Altamirano et al (2004) through which different microalgae genotypes render distinct sensitivities to a particular toxicant. This concept can be applied on Dc^{CdR100} strain, which used in tandem in conjunction with the wild type strain (Dc^{wt}) would allow its potential application in a dual-head biosensor system for detecting Cd. Wild type *D. chlorelloides* (Dc^{wt}) strain exhibited high sensitivity to Cd whereas Dc^{CdR100} strain exhibited high resistance to Cd. Thus, Dc^{wt} strain would provide the biosensor sensitivity while its specificity arises from the different Dc^{CdR100} strain responses (Figure 4).

Although the practical advantages of biosensors are well known, their measurements should be verified and validated before being accepted. In this sense, some of the aspects described by Thevenot et al. (2001), such as sensitivity and detection limits, have been studied in this work through the photosynthetic activity exhibited by Dc^{wt} and Dc^{CdR100} strians. Thus, the results showed that Dc^{CdR100} has reduced its Φ_{PSII} activity about 65% compared to Dc^{wt}. This difference between the two strains could be used specifically for their inclusion into a dual head biosensor.

These results are consistent with the view taken by Schreiber et al. (2002) by which Φ_{PSII} is an excellent biomarker of risk associated with toxic pollutants on aquatic microalgae. This method provides no information on the photosynthetic performance of a sample to the continuous light. The quantum yield is affected by many more contaminants than those inhibiting the primary reactions near to photosystem II. So, because the specific Φ_{PSII} rate is considered a reproducible ecologically relevant response and not strongly test system specific, it was selected as an appropriate test endpoint.

In the present study, photosynthetic oxygen development and respiration response exhibited by Dc^{CdW100} strain and differences compared to Dc^{wt} strain were also studied, and the results showed that the inhibition percentage measured on gross photosynthesis values were lower than those obtained on Φ_{PSII} activity, being higher the differences in dark phase (respiration) than light phase (net photosynthesis). These results indicate greater activity stability of the activity of photosystem I (PSI). As shown in some other studies, PSI showed the ability to maintain physiological activity under environmental stress (Li et al., 2006; Huang et al., 2010). Thus, these results showed the tolerance of PSI to Cd exposure. On the contrary, PSII was more susceptible to Cd exposure.

Many studies demonstrated that PSII was one of the most sensitive target sites for environmental stress, such as heavy metals (Qian et al., 2009; Zhang et al., 2010). Huang et al. (2010) demonstrated that exposures to 100 µM Cd had no inhibition on Φ_{PSI} whereas effective Φ_{PSII} showed significant decrease compared to the control. Results obtained by Atal et al. (1991) suggested that Cd caused the disassembly of PSII, and Tukaj et al. (2007) reported that that Cd seriously inhibited oxygen evolution.

Some authors demonstrated the occurrence of Cd content as intramitochondrial granules, coupled with functional and structural changes, in selected strains of green algae exposed to heavy metal (Silverberg 1976). These effects was in accordance with the inhibition of respiratory O_2 consumption rate (Figure 4). Thus, modification of cell wall, cytoplasmic vacuolization and the degradation of chloroplast were the most striking modifications. These results suggest the localization of Cd in the cells and the role of cell wall and vacuole in conferring metal tolerance (Aguilera and Amils, 2005; Kola and Wilkinson, 2005)

Several authors have shown in their studies the ability of algae to absorb heavy metals (Megharaja et al., 2003) and monitor their concentration in aquatic environments (Sigg, 1987). This high capacity to sequester heavy metals from the environment is linked to bioaccumulation processes.

Our results reveal that much of Cd added to *D. chlorelloides* cultures appears bound to the cell wall. This adsorption process would be consistent with the fact that the cell wall is a first wall in the absorption of the heavy metal and plays a key role in the processes of tolerance to metal toxicity. Macfie and Welbourn (2000) reported that total amount of Cd uptaken by a walled-strain of *Chlamydomonas reinhardtii* was greater than the wall-less strain after 24 h.

Is widely known as an effort to protect themselves against metal toxicity, microalgae have developed mechanisms other than the adsorption phenomenon. Thus, although Dc^{CdR100} mainly accumulates Cd in cell walls, approximately 30% of removed Cd intracellularly appears. These results are in agreement with those obtained by other authors. Albergoni et al. (1980) demonstrated that the single-celled flagellate *Euglena glacilis* chelated and accumulated the

higher amount of cadmium into the cells. In the same way, Carr et al. (1998) observed that the green algae *Chlorella vulgaris* accumulated Cd intracellularly.

Conclusion

In summary, wild-type *D. chlorelloides* populations previously exposed to Cd causes the appearance of resistant cells by means rare spontaneous mutation to this specific heavy metal. These cells exhibit both morphological and photosynthetic activity changes compared with wild type cells, which can be exploited to obtain a specific microalgal biosensor able to determine cadmium in freshwater environments. Additionally, Dc^{CdR100} strain was found to have good accumulation properties for Cd. The high amount of Cd in its cells and its high Cd tolerance suggest the possibility of using these microalgae strains in bioremediation processes on freshwater polluted with this heavy metal.

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Table 1. Results obtained by mean chlorophyll fluorescence measurements with the dual-channel PAM fluorometer ToxY-PAM. Values represent mean±sd of eight independent assays (n=8).

		ToxY-PAM					
***D. chlorelloides* strains**		**Channel 1 (sample channel)**			**Channel 2 (reference channel)**		
sample vs reference	**n**	**F1**	**Fm1**	**Y1**	**F2**	**Fm2**	**Y2**
Dc^{wt} vs Dc^{wt}	8	1475.22±8.56	1750.33±14.65	0.16±0.01	1354.90±9.11	1604.52±16.37	0.16±0.01
Dc^{wt} vs Dc^{CdR100}	8	1469.46±5.97	1737.29±6.65	0.15±0.01	1093.15±93.30	1995.17±161.60	0.44±0.01***

F1, F2: fluorescence yields measured shortly before application of a saturation pulse in Ch1 and Ch2, respectively.

Fm1, Fm2: maximal fluorescence yields measured during a saturation pulse in Ch1 and Ch2, respectively.

Y1, Y2: effective quantum yields of the samples in Ch1 and Ch2, respectively.

***: significant differences ($p<0.0001$) respect to Y1.

Table 2. Oxygen balance (Pg) exhibited by the wild type Dc^{wt} and Dc^{CdR100} strains and measurement of oxygen production (light phase, Pn) and consumption (dark phase, R) by both *D. chlorelloides* strains. Values represent mean±sd of eight independent assays (n=8).

		***D. chlorelloides* strain**		
photosynthesis parameter	**n**	**Dc^{wt} (nmol O_2 ml^{-1} min^{-1})**	**Dc^{CdR100} (nmol O_2 ml^{-1} min^{-1})**	**inhibition (%)**
Pg	8	72.39±3.74	50.81±5.31**	29.95±3.91
Pn	8	61.62±2.40	45.98±3.95**	25.64±3.59
R	8	-10.77±1.38	-4.83±1.36**	55.81±7.52

Pg: gross photosynthesis; Pn: net photosynthesis; R: respiration.

**: significant differences ($p<0.001$) respect Dc^{wt} values.

Table 3. Cadmium removed by the 30th day in cultures of Dc^{CdR100} strain exposed to Cd 100 µM. Values represent mean±sd of eight independent assays (n=8).

initial Cd^{+2} added to cultures (µM)	**intracellular Cd^{+2} (µM)**	**bioadsorbed Cd^{+2} on cell surface (µM)**	**Cd^{+2} unremoved (µM)**
100	29.18±4.67	62.45±5.22	8.36±1.23

Figure 1.

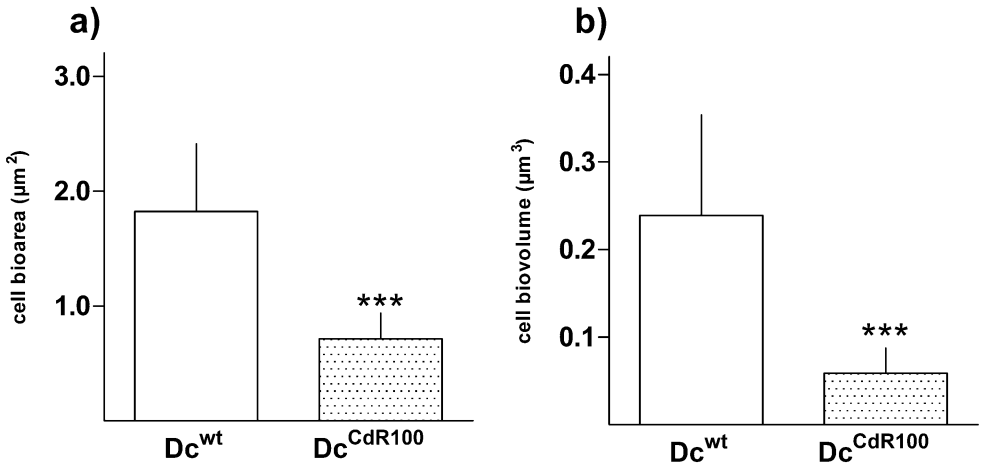

Figure 2.

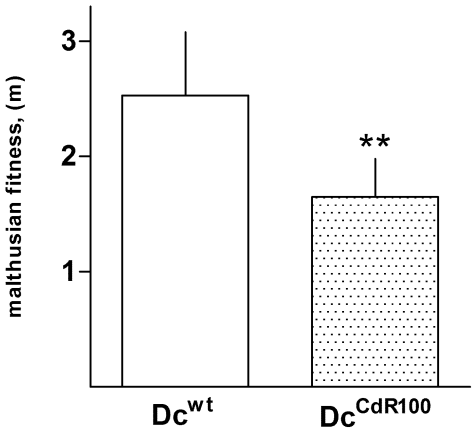

Figure 3.

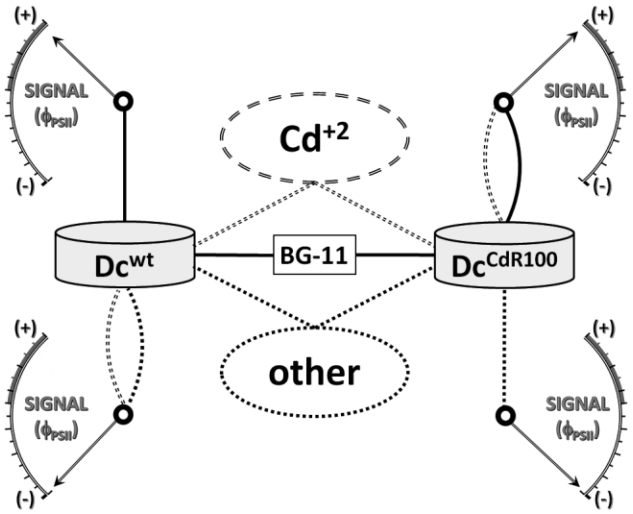

Figure 4.